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Review

Post-translational protein translocation into thylakoids by the Sec and ΔpH-dependent pathways

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Abstract

Two distinct protein translocation pathways that employ hydrophobic signal peptides function in the plant thylakoid membrane. These two systems are precursor specific and distinguished by their energy and component requirements. Recent studies have shown that one pathway is homologous to the bacterial general export system called Sec. The other one, called the ΔpH-dependent pathway, was originally considered to be unique to plant thylakoids. However, it is now known that homologous transport systems are widely present in prokaryotes and even present in archaea. Here we review these protein transport pathways and discuss their capabilities and mechanisms of operation.

Keywords: Tat; Twin arginine; Folded protein transport; Chloroplast; Amphipathic helix; Signal peptide

1. Introduction

The thylakoid, a closed internal membrane system in chloroplasts, is a photosynthetic membrane that converts light energy to chemical energy and reducing power. Photochemical reactions are performed by several thylakoid supercomplexes. Components and biogenesis factors for these supercomplexes are encoded in both nuclear and plastid genomes. As a consequence of this dual genetic origin, the assembly of nuclear-encoded thylakoid proteins starts with appropriate targeting from the cytosol; assembly of plastid-encoded thylakoid proteins begins in the stroma or on the membrane.

Nuclear encoded thylakoid proteins are synthesized in the cytosol as precursors with cleavable transit peptides (Fig. 1A). Precursor proteins are first imported into the chloroplast stroma, a step governed by the transit peptide and accomplished by the chloroplast general import machinery [1]. From the stroma, intermediate precursors are translocated into or across the thylakoid membrane by at least four distinct pathways: the Sec, ΔpH-dependent, SRP and spontaneous systems. The Sec and ΔpH-dependent pathways transport soluble proteins to the thylakoid lumen and also integrate thylakoid membrane proteins (Table 1). The SRP-dependent and spontaneous systems appear to be specific for integral membrane proteins. Precursors transported by the Sec, ΔpH-dependent, and frequently the spontaneous pathways possess bipartite transit peptides with an N-terminal stroma-targeting domain (STD) and a C-terminal lumen-targeting domain (LTD). As will be discussed, the LTDs consist of signature tar-

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geting elements for prokaryote-derived protein export systems and reflect the translocation machinery that mediates recognition and translocation. This review focuses on the protein transport by the chloroplast Sec and ΔpH-dependent pathways. The other thylakoid pathways are also reviewed in this issue. Additional information and perspectives are available in several reviews on thylakoid protein transport and bacterial protein export systems [2–9].

2. Two independent pathways

In vitro protein transport assays with isolated thylakoids made it possible to manipulate energy and stromal protein availability [10,11]. Assays with various precursor proteins revealed that there are two independent protein translocation pathways that could be distinguished by their requirements [11] (Table 1 and Fig. 1A). Translocation of a subset of precursors is absolutely dependent on ATP and stromal extract and is stimulated by the thylakoidal ΔpH [10,12–15,17]. Translocation of a second subset of precursors requires the thylakoidal pH gradient, but neither stromal extract nor ATP [11,15,30]. Competition studies with chemical amounts of *Escherichia coli*-overexpressed precursor proteins confirmed that precursors are targeted to two distinct transport pathways and produced the same substrate groupings [21]. Two signal peptide-dependent protein transport pathways were also genetically shown in maize, where the thylakoid assembly mutant *tha1* and high chlorophyll fluorescence mutant *hcf106*, respectively, displayed impairment of transport of essentially the same two subgroups of proteins (Table 1) [22]. Identification of components (see below) showed that the thylakoid ATP-dependent pathway is homologous to the *E. coli* Sec pathway and revealed that the ΔpH-dependent pathway is homologous to a bacterial protein transport pathway called the 'Tat', twin arginine translocation or 'MTT', membrane targeting and translocation pathway [23–25]. As will be discussed below, additional studies verify the independence of the Sec and ΔpH-dependent/Tat pathways.

2.1. Pathway selection of precursors; distinctive signal peptides for selectivity and mature domains for efficiency

As mentioned, the Sec and ΔpH-dependent pathways are specific for subgroups of precursor proteins (Table 1). No genuine precursor targeted for the both pathways has been found, although recombinant precursors [26] and the cyanobacterial CtpA [27] can be targeted to both pathways in vitro. In thylakoids, Sec and ΔpH-dependent pathways appear to transport roughly equivalent numbers of substrates (Table 1) [28]. In contrast, substrates of the bacterial Tat system represent only a fraction (~3% in *E. coli*) of the precursors transported by the Sec system [29]. Targeting of the two sets of thylakoid precursors is specified by the LTDs. These domains have features of hydrophobic signal peptides of bacteria, which include a charged N-terminal region (N), a hydrophobic core region (H), and polar C-terminal region (C) with an A-X-A motif for cleavage by the thylakoid processing peptidase (Fig. 2). In addition, most lumen-targeting domains possess amino proximal A domains that usually contain acidic residues, which are uncommon in transit peptides. The function of the A domains is unknown as their deletion does not impair transport in vitro [26]. Domain-swapping experiments and mutagenesis studies showed that pathway specificity is determined by subtle differences between LTDs [30–32]. The thylakoid pathway targeting elements are apparently conserved with prokaryotes because signal peptides from two different Tat pathway precursors specified exclusive transport by the thylakoid ΔpH-dependent pathway in vitro [33,34].

Table 1
Identified precursor proteins targeted to the Sec or ΔpH-dependent pathways

Pathways	Thylakoid lumen	Thylakoid membrane
Sec	Plastocyanin [12], OE33 [10]	Cytochrome *f*[a] [16], PSI-F [14]
ΔpH	OE17 [11], OE23 [11], Hcf136 [19], P16 [20], PSI-N [18], PSII-T [30]	Pftf [81]

[a]Cytochrome *f* is encoded in the plastid genome and synthesized with signal peptide.

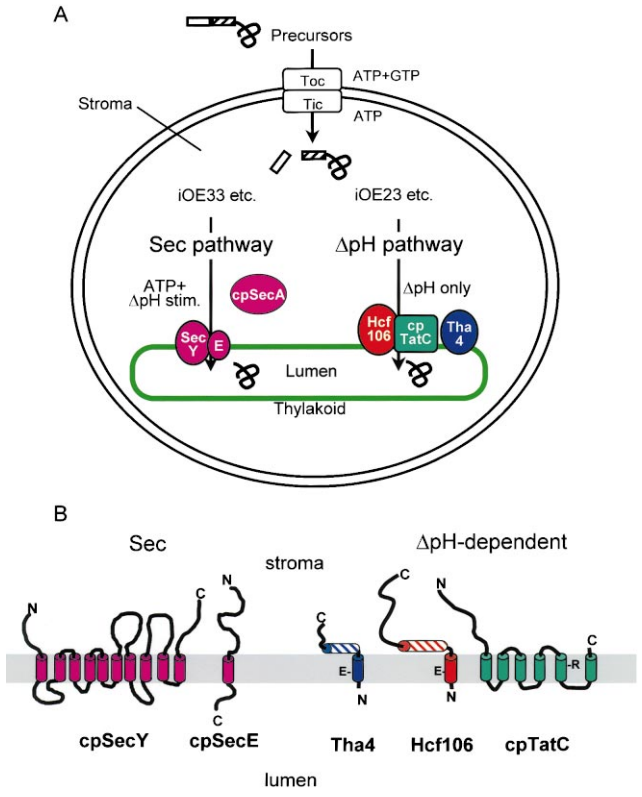
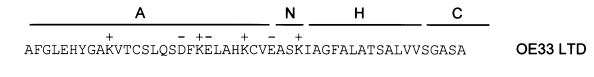


Fig. 1. Pathway for localization of nuclear-encoded thylakoid proteins via the Sec or ΔpH-dependent pathways. (A) Cytosolically-synthesized precursors are imported into the chloroplast stroma via the Toc and Tic complexes of the general import pathway. This step is governed by the stromal targeting domain of the transit peptide (open box), which is removed by the stromal processing peptidase. Intermediate precursors are then transported into thylakoids by the Sec or ΔpH-dependent pathway, a step governed by the lumen-targeting domain of the transit peptide (cross-hatched box). The components and energy requirements of the two pathways are shown. See Table 1 for a list of documented substrates of the two pathways. (B) Topology of membrane components of Sec and ΔpH-dependent pathways. Membrane-spanning helices are drawn as solid color cylinders. Predicted amphipathic helices of Hcf106 and Tha4 are drawn as cross-hatched cylinders. Positions of conserved glutamic acids (E) in N-proximal membrane-spanning helices of Hcf106 and Tha4 and conserved arginine (R) in the fifth membrane-spanning helix are indicated.

A twin arginine motif in the N-domain is a characteristic of all ΔpH-dependent precursors and distinguishes them from Sec pathway precursors. In bacterial Tat precursors, the twin arginine is found in loosely conserved motif, [ST]-R-R-X-F-L-K [29]. The importance of the RR was shown by mutating the twin arginine motif to QQ, RK, KR or KK, each of which dramatically impairs the ability of the precursor to be transported [26,35]. Similar studies in bacteria have shown the importance of the twin arginine for Tat precursor proteins [36–38].

Two observations indicate that signal peptides for the ΔpH-dependent pathway have an additional specificity determinant in their H/C domains. First, a chimeric signal peptide that contains the N-domain from a ΔpH-dependent precursor fused to the H/C-domains from a Sec pathway precursor can direct certain passenger proteins to both pathways [26]. Second, substitution of the RR motif to KR, RK, or KK did not convert the signal peptide to a Sec directing signal peptide [35]. This additional determinant has been labeled 'Sec-avoidance'. Two different hypotheses for Sec-avoidance or incompatibility have been offered based on studies of a limited number of precursor proteins. One is that basic residues in C-domains of ΔpH-dependent LTDs, when combined with the RR, are incompatible with the Sec pathway [39]. The other hypothesis proposes that characteristics of the H-domain of ΔpH-dependent precursors make them incompatible with the Sec pathway [26]. Logoplot analysis of thylakoid [28] and bacterial [40] signal peptides found that ΔpH-dependent/Tat signal peptides have a lower frequency of hydrophobic residues in their H-domains. However, neither hypothesis is generally applicable because basic residues are not conserved among ΔpH-dependent signal peptides

A



B

LTD	A-domain	N-domain	H-domain	C-domain
Sec	Variable length, usually contains acidic residues	Charged residues	~15 residues	Absence of basic residues
ΔpH	Variable length, usually contains acidic residues	Twin arginine	12-18 residues On average, less hydrophobic than Sec	Frequently contains basic residue(s)

Fig. 2. Lumen-targeting domains of thylakoid Sec and ΔpH-dependent precursors. (A) Typical lumen-targeting domains of Sec (OE33) and ΔpH-dependent (OE23) precursors. Regions of amino-proximal acidic (A), charged (N), hydrophobic core (H) and a polar C-terminal region (C) are indicated. Charged amino acid residues are indicated by '+' or '−'. (B) Features of each region of known Sec and ΔpH-dependent precursors (see Table 1).

[28] and because on an individual basis there is considerable overlap in peak and total hydrophobicity between ΔpH-dependent and Sec signal peptides [26]. A systematic approach may be necessary to glean the essential elements for ΔpH-dependent/Tat pathway targeting. At present, one can only say with confidence that exclusive targeting to the ΔpH-dependent pathway requires a twin arginine motif in the N-domain and an H/C domain that is incompatible with the Sec pathway. Exclusive targeting to the Sec-dependent pathway is achieved by a charged N-domain that lacks the twin arginine motif and a compatible H/C domain.

The mature domain of precursor proteins contributes to pathway compatibility. For example, even when equipped with Sec signal peptides, mature protein domains of precursors normally directed to the ΔpH-dependent pathway are not efficiently transported by the Sec pathway [26,30,41]. The converse is not true because at least two Sec pathway substrates are efficiently transported by the ΔpH-dependent pathway when equipped with a suitable signal peptide [26,30,31]. This may be related to the observation that ΔpH-dependent precursors are folded prior to transport (see below). One possibility is that the Sec pathway is unable to unfold these precursors. Another possibility is that the folded domain interferes with proper presentation of the signal peptide to the Sec machinery. In support of the signal peptide-accessibility model is the fact that the recombinant precursors DT23 and DT17, which possess the Sec-compatible DT (dual targeting) signal peptide and ΔpH pathway passenger proteins, fail to form a complex with cpSecA and cpSecY on thylakoids (Ma and Cline, unpublished). Furthermore, DT23 is unable to competitively inhibit Sec pathways transport, even though it is a very efficient competitor of ΔpH pathway transport (Ma and Cline, unpublished).

The ΔpH-dependent pathway is also sensitive to the mature domain of the precursor and may possess proofreading capability. Theg and co-workers found that a C-terminally truncated pOE23 is poorly translocated into the lumen [42]. Biotinylation of the iOE23 carboxyl proximal cysteine (Henry and Cline, unpublished) or biotinylation and avidin-binding to the pOE17 C-terminus [43] also prevented translocation. This block cannot be easily explained as resulting from malfolding because the ΔpH-dependent pathway seems able to transport unfolded or malfolded precursor proteins. Specifically, incorporation of amino acid analogs into pOE23, which causes destabilization of OE23 mature domain, did not block its translocation into the lumen [44]. The bacterial Tat system appears to have a similar proofreading function because inhibition of cofactor insertion into Tat-dependent precursors blocks export of the precursor and results in its accumulation in the cytoplasm [25]. It has been speculated for Tat substrates that the basis for this proofreading is that signal peptides are buried until protein folding and assembly are complete [7]. This appears not to be the case for thylakoids because the above-mentioned biotinylated precursors both effectively compete for transport of other precursors, indicating that their signal peptides are recognized by the machinery (Henry and Cline, unpublished) [43].

2.2. Components of the systems

2.2.1. Sec

Thylakoid Sec components (Fig. 1) were identified by a homology-based approach. For example thylakoid transport of OE33, plastocyanin and PS1-F is sensitive to azide, which is known as an inhibitor of the bacterial SecA protein [14,30,45]. Genes homologous to bacterial SecA were found in algal plastid genomes [46,47] and a SecA was purified from pea chloroplasts using an antibody raised to an algal SecA peptide [48]. Pea cpSecA substitutes for stromal extract to support OE33 and plastocyanin transport into thylakoids in azide-sensitive manner [48]. Preincubation of stromal extract with anti-cpSecA also inhibited OE33 transport [49]. cpSecA was also identified independently by a genetic approach in which the maize mutant *tha1* was found to result from disruption of the maize cpSecA gene [50].

A chloroplast SecY homolog was identified in the *Arabidopsis* EST collection [51]. Antibodies made to the cpSecY C-terminal peptide inhibit SecA-dependent protein translocation, but not ΔpH-dependent or post-translational SRP-dependent protein translocation [52,53]. A gene homologous to bacterial SecE was also found in *Arabidopsis* genomic sequence. The *Arabidopsis* SecE is an integral thylakoid protein with one membrane-spanning domain and forms a

180 kDa complex with chloroplast SecY [53]. There is no direct evidence for the function of cpSecE in thylakoids, but its association with cpSecY suggests involvement in Sec pathway transport.

The SecYE complex and SecA are a minimum entity to perform protein translocation in the bacterial Sec system [54,55]. Bacterial SecB is a molecular chaperone that prevents precursor proteins from folding into structures incompatible with translocation [56]. Bacterial SecA has a C-terminal 20-residue sequence that binds to SecB [57]. Chloroplast SecA lacks an analogous sequence and a SecB gene appears to be absent from the completely sequenced *Arabidopsis* genome. In *E. coli*, SecG is complexed with SecY and SecE and stimulates protein translocation under certain conditions [58]. In addition, a trimeric complex of SecD, SecF and YajC interacts with SecYEG [59] and stabilizes the membrane-inserted state of SecA [60]. There are no genes homologous to secG, secD, secF or yajC in the *Arabidopsis* genome. Thus, the plant thylakoid Sec system appears to operate with the minimum number of required components. However, the possibility of component(s) unique for the thylakoid Sec pathway cannot presently be ruled out. Of interest is that a second and significantly diverged chloroplast SecY (accession number AC007071) is predicted from *Arabidopsis* genomic sequence. The function of this protein and its location remain to be examined.

2.2.2. ΔpH-dependent

Three components of the ΔpH-dependent pathway, Hcf106, Tha4 and cpTatC have been genetically and biochemically identified ([52,61,62,86] (Fig. 1B). The *E. coli* orthologs are TatB, TatA/E and TatC, respectively [23,63]. Hcf106 and Tha4 are homologous proteins with similar structures; they are anchored to the membrane by an amino proximal transmembrane domain and expose a predicted amphipathic helix and an acidic C-terminal domain to the stroma. The Hcf106 C-terminal domain and amphipathic helix are notably longer than those of Tha4. The highest degree of sequence similarity between Hcf106 and Tha4 is in the amino proximal regions, especially in the transmembrane domain (~65% identity). cpTatC is an integral membrane protein with six membrane-spanning helices and its amino and carboxyl termini exposed to the stroma, which corresponds to the cytoplasm of bacteria [86]. Each of these components appears to play a direct role in protein transport because treatment of pea thylakoids with antibodies to any single component specifically disables the ΔpH-dependent pathway ([52,86].

The *E. coli* orthologs to these components are similar in structure and sequence and are presumed to adopt the same topology in the membrane. Only one point mutation of Tat system components has been identified. Substitution of a proline for leucine in the predicted hinge region between the membrane-spanning helix and the amphipathic helix of TatB impaired Tat pathway transport (Fig. 3) [24]. The proline residue and preceding glycine residue are completely conserved among Hcf106 as well as TatB proteins (Fig. 3), suggesting that a flexible interface between the membrane-spanning helix and amphipathic helix plays an essential conserved role. Other comparisons among components of the different systems identify conserved motifs, suggestive of important functional roles. A proline–glutamic acid motif in the transmembrane domain is also strictly conserved among Hcf106 and Tha4 proteins; TatBs have a conserved glutamate in the corresponding position (Fig. 3). As there is a considerable energy cost to maintaining a charged residue in a transmembrane domain, these glutamate residues are likely to play important role(s) in the transport process. TatB and TatC are now known to interact with each other in the membrane (see below). One function of the Hcf106/TatB glutamate may be to stabilize cpTatC/TatC via interaction with a conserved transmembrane arginine residue. Interestingly, TatA/E proteins generally have a lysine, histidine, or glutamine residue at the position corresponding to the glutamate of Tha4 (Fig. 3), suggesting that Tha4 and TatA/E proteins play somewhat different roles in transport. In contrast to the conserved regions, the C-terminal acid domain of Hcf106 homologous proteins varies in length and also lacks sequence conservation. The finding of Weiner et al. in 1998 that a truncated TatB lacking the C-terminal domain complemented the TatB hinge mutant described above suggests a nonessential role for the acidic C-terminal domains [24].

Whether other components participate in ΔpH-dependent/Tat transport is presently unknown. However, several observations are consistent with the

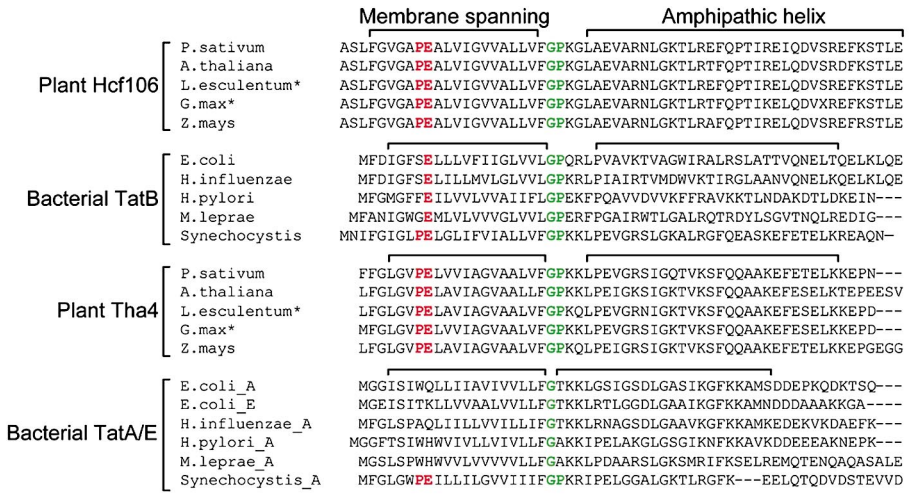


Fig. 3. Sequence alignment of plant Hcf106 and Tha4 and bacterial TatA, B and E proteins. The predicted transmembrane domains and amphipathic helices of plant Hcf106 and Tha4 proteins and bacterial TatA, B and E proteins were aligned with the ClustalW 1.8 program. Chloroplast targeting peptides of the plants proteins and the acidic C-terminal domains are not shown. The conserved proline–glutamate (or glutamate) motif in the membrane spanning helix is colored in red. The conserved glycine–proline (or glycine) motif in the predicted hinge region between the membrane-spanning helix and the amphipathic helix are colored in green. Hcf106 and Tha4 sequences of *Glycine max* and *Lycopersicon esculentum* are translated from EST clones. *Haemophilus influenzae*, *Helicobacter pylori*, *Mycobacterium leprae* and *Synechocystis* do not encode a TatE protein.

idea that these are the only components. Berks et al. note that while genes for TatC and TatA/B/E show genetic linkage in many cases, no uncharacterized genes consistently linked to the Tat genes are apparent in genomes of sequenced prokaryotes [7]. Second, the purified *E. coli* 600 kDa Tat complex contains only TatA, TatB, and TatC [64], and a partially purified precursor–700 kDa cpTatC–Hcf106 complex (see below) appears to contain largely, if not exclusively, cpTatC, Hcf106, and precursor (Mori and Cline, unpublished). Third, recent work by Wicker's group found that overexpression of the TatA/B/C operon upregulated Tat pathway transport [65]. Nevertheless, this point requires further study.

2.3. Capabilities and mechanisms

2.3.1. Sec pathway

The limited number of studies on the thylakoid Sec pathway indicates that it operates similarly to the well-characterized bacterial Sec pathway [6]. In the bacterial system, proteins bind to SecA at the membrane and are threaded across the bilayer in a largely unfolded conformation (see [3] for review). Upon binding ATP, SecA inserts across the bilayer, carrying 2–2.5 kDa of precursor protein with it. Upon ATP hydrolysis, SecA releases the precursor protein and deinserts from the membrane. The proteins cross the membrane through a channel made up in part by the SecYE complex. Reiteration of the SecA insertion–deinsertion cycle results in translocation of the entire substrate protein. Sodium azide inhibits deinsertion of SecA from the membrane by inhibiting ATP hydrolysis [66]. The proton motive force optimizes the transition of SecA from its inserted to its deinserted state to stimulate the protein translocation cycle [67]. Similarly, thylakoid precursor proteins bind to the membrane in a cpSecA-dependent manner and form a complex on the membrane that also contains cpSecY (Ma and Cline, unpublished). Tightly folded proteins are not transported by the thylakoid Sec system [44]. Bacterial and thylakoid Sec systems transport soluble lumenal proteins as well as integrate membrane proteins (Table 1). Integration of proteins in bacteria appears to result from the partitioning of transmembrane domains out of the aqueous channel and into the bilayer [68]. In bacteria, SecA appears to function primarily in translocation of larger hydrophilic regions that flank signal peptides or hydrophobic signal-anchors on the carboxyl proximal side [8]. At present, it is not known if the thylakoid system operates in this fashion. However, known thylakoid

Sec substrates fit this model. In summary, proteins transported or integrated via the Sec machinery must be easily unfolded during translocation. In addition, Sec pathway substrates that function with cofactors must assemble with their prosthetic groups in the lumen rather than the stroma. Examples of such Sec substrates are plastocyanin and cytochrome *f* [69].

2.3.2. ΔpH-dependent/Tat system

Much less is known regarding the operation of the ΔpH-dependent and homologous bacterial Tat systems, but it is clear that these systems complement Sec in capabilities. They possess two striking characteristics: the ability to transport folded proteins and the sole use of the pH gradient as energy source (for thylakoids). The ΔpH-dependent system has been directly shown to transport folded polypeptides with diameters of ~2–3 nm. A chimeric precursor with an internally crosslinked bovine pancreatic trypsin inhibitor (BPTI) fused to the C-terminus of the precursor pOE17 was efficiently translocated across the ΔpH-dependent pathway [70]. In a similar experiment, a chimeric protein in which mouse dihydrofolate reductase (DHFR), whose tight folding is caused by the folate analogs methotrexate or aminopterin, was linked to C-terminus of pOE23. pOE23-DHFR was translocated on the ΔpH-dependent pathway, even in the presence of aminopterin [44].

Documented substrates of the thylakoid system range in size from 3600 to 36 000 Da. The presence of a twin arginine in its signal peptide suggests that polyphenoloxidase, ~60 000 Da, is also translocated by the ΔpH-dependent system, but this remains to be definitively shown [71]. The conformation of authentic substrates during transport has not been established. Indirect evidence suggests that iOE23 and iOE17 are tightly folded when presented to the transport machinery [43,72], but it is unclear if they remain so during transport. If these substrates are all folded during transport, a putative channel would have to expand to ~5 nm. Most of the substrates of the bacterial Tat system are cofactor-binding proteins that are assembled with their prosthetic groups in the cytoplasm. These proteins range in size, but are estimated to present the translocation system with diameters up to 7 nm, which is larger than the thickness of the bilayer [7]. The bacterial hydrogenase 2 is possibly the most interesting substrate of the Tat pathway. It is composed of a small subunit that contains a Tat signal peptide and a large subunit that lacks an export signal. These two subunits are cotranslocated into the periplasm [73]. Interestingly, neither subunit is translocated in the absence of the other. The manner by which these systems translocate proteins in folded state and at the same time maintain the membrane in an ion-tight state [74] is an intriguing puzzle.

Biochemical studies in plants and genetic studies in bacteria are beginning to reveal some aspects of the mechanisms of these systems. In thylakoids, the process has been broken down into a binding step and a translocation step. Binding occurs in the absence of the pH gradient; the bound precursor is translocated to the lumen when the ΔpH is applied [43,75]. This binding step appears to be the precursor recognition step for this pathway. It has been speculated that the ΔpH system employs a precursor receptor because the consensus RR motif is unique among classical signal peptides. Hcf106/TatB [61] and Tha4/TatA [76] have been proposed as receptors because of their receptor-like topology. TatC/cpTatC has been suggested as the receptor because it is more conserved among species than other components [7].

Experimental results in our lab have identified the precursor-binding site as a large complex containing both cpTatC and Hcf106. Antibody inhibition studies implicated both cpTatC and Hcf106 in the binding process. In resting state thylakoids, cpTatC and Hcf106 are present in an ~700 kDa complex as assessed by blue native gel electrophoresis (BN-PAGE) and coimmunoprecipitation of digitonin-solubilized membranes [87] (Fig. 4). A portion of the total Hcf106 and all of the Tha4 are present in other complexes. This component organization was confirmed by in situ cross-linking experiments with intact thylakoids. When precursors bind to thylakoids, they can be recovered on BN-PAGE in association with the ~700 kDa cpTatC–Hcf106 complex and several lines of evidence indicate that Tha4 is absent from the precursor bound complex. Precursor binding to the cpTatC–Hcf106 complex is strictly dependent on the twin arginine motif of the LTD as well as the hydrophobic core, both of which are crucial factors for protein translocation on the ΔpH-dependent pathway [26]. Thus, the recognition site could consist of a hydrophobic pocket or groove anchored with acidic or polar residues to stabilize the

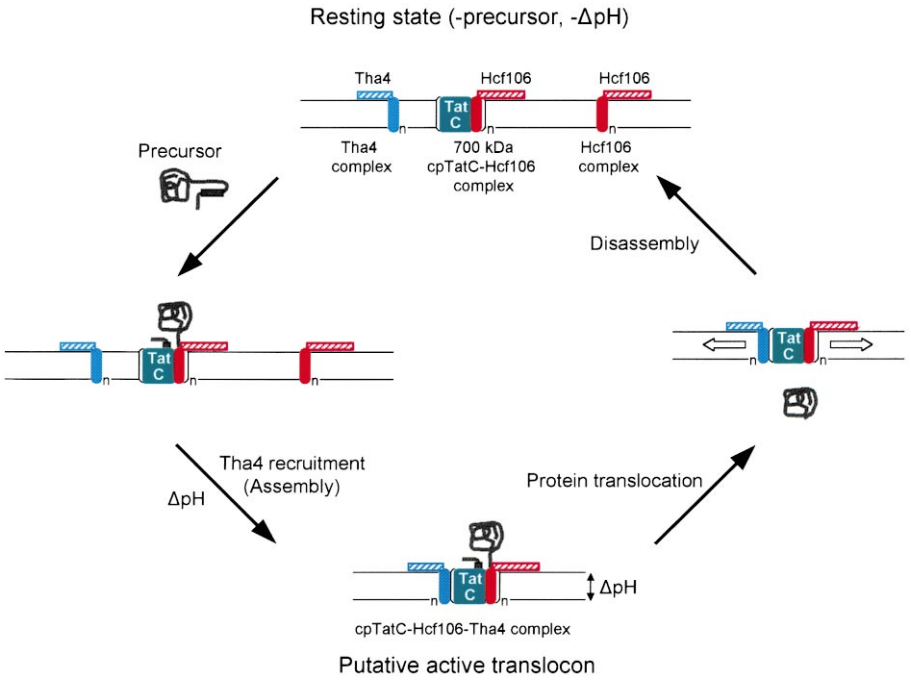


Fig. 4. Working model for assembly of the ΔpH-dependent pathway translocon. In thylakoids at rest, i.e., the absence of precursor and ΔpH, cpTatC and Hcf106 exist in a ~700 kDa complex that appears to consist of multiple copies of cpTatC–Hcf106 heterodimer (see text). All of Tha4 and a portion of Hcf106 are present in separate complexes. Precursors bind to the cpTatC–Hcf106 complex and, upon establishment of the pH gradient, Tha4 is recruited to the cpTatC–Hcf106-precursor complex. Translocation occurs and the translocon disassembles.

guanidino groups of the arginines. Because Hcf106 uncomplexed with cpTatC appears not to bind the precursor [87], acidic residues on stromal-facing regions of cpTatC are likely candidates. Two conserved motifs containing invariant glutamic acids are found among all plant and bacterial TatC homologs. The first is H-[IL]-x-E-[FIL]-[KR]-x-R just prior to the first membrane-spanning helix and the second is P-[AG]-[LM]-x-x-x-E-[KRT]-[KRNG] in the short connection loop between second and third membrane-spanning helices of TatC. These motifs, especially the glutamate residues, are likely targets for mutagenesis studies.

A key question is 'what happens to component organization when the pH gradient is restored'? Several observations provide indirect evidence that Tha4 joins the large complex and that this leads to formation of an active translocon. First, antibody inhibition experiments show that Tha4 acts downstream of precursor binding. Specifically, prebinding of anti-Tha4 IgG to thylakoids does not impair precursor binding, but inhibits translocation of the bound precursor [75]. Second, kinetic analysis of precursor transport from the bound state indicates that a rate-limiting step occurs on the membrane after the pH gradient is imposed [43,75]. Musser and Theg additionally showed that proton transfer limits events on the membrane leading to translocation [77]. This would be consistent with such a hypothetical ΔpH-triggered assembly process. Finally, the fact that the purified ~600 kDa *E. coli* Tat complex contains a small amount of TatA (Tha4 ortholog) in addition to the large amounts of TatC and TatB (Hcf106 ortholog) suggests that under appropriate conditions, all three components can be present in one complex [64]. Analysis of the components in the process of translocation will be necessary to test this hypothetical assembly step. This might be achieved by trapping a translocation intermediate. Klösgen and coworkers [78] reported on a putative translocation intermediate of the ΔpH-dependent pathway, wherein the precursor protein appeared to be largely translocated into the lumen but still remained associated with the membrane. This inter-

mediate migrated on blue native gel electrophoresis at approximately 560 and 620 kDa, which is similar in size to the complex that we have seen, although the composition of the 620 and 560 kDa complexes remain to be determined. It is possible that these complexes represent active translocons. However, it may also be that they are analogous to the cpTatC–Hcf106-precursor complex that we have detected because the reported intermediate appears to be tethered to the membrane by an uncleaved signal peptide, possibly still bound to the receptor site.

Based on these results and assumptions, we propose a cyclical assembly model for the components of the ΔpH pathway (Fig. 4). In the resting state, with no precursor and no thylakoidal pH gradient, 700 kDa cpTatC–Hcf106 and Tha4 complexes are separately present in the membrane (Resting state). Precursor binds the 700 kDa cpTatC–Hcf106 complex. This step could be reversible or weak for most genuine precursor (Binding step). The pH gradient triggers Tha4 recruitment to the precursor-bound cpTatC–Hcf106 complex and causes a rearrangement of components to form an active translocon (Assembly step). Upon completion of protein translocation, Tha4 is released, and cpTatC and Hcf106 reform the 700 kDa complex (Disassembly step). It is likely that Hcf106 and cpTatC remain associated throughout the process as a TatB–TatC fusion protein functionally complements a null mutant lacking both TatB and TatC in *E. coli* [64].

Such component association could be linked to formation of an active translocon. Several different models have been proposed for the mechanism of folded protein translocation. These include an endocytic process, a gated channel, and a dynamic flexible channel [79]. We recently produced a putative translocation intermediate in which large protein domains span the membrane via a peptide linker (Fincher and Cline, in preparation). This is most consistent with the flexible channel model. Berks et al. have suggested that multiples of Tha4/TatA and/or Hcf106/TatB assemble around a core cpTatC/TatC to form the channel [7]. Expansion and contraction would be accomplished by adding or removing Hcf106 and/or Tha4 monomers. Quantitative immunoblotting shows that cpTatC is present in thylakoids at about 18 000 copies per chloroplast [86], similar to the estimated number of ΔpH-dependent pathway translocation sites [80], whereas Hcf106 and Tha4 are present at 5–10-fold the number of translocation sites. This stoichiometry is consistent with the dynamic channel model for a membrane that transports proteins up to ∼5 nm. Models for the structure of the translocon must allow for insertion of transmembrane domains because at least one integral membrane protein, Pftf, employs the ΔpH-dependent pathway for its integration [81]. In the flexible channel model, transmembrane domains might enter the lipid bilayer by passing between the monomeric subunits of the channel wall.

It has also been suggested that the amphipathic helices of Tha4/TatA and Hcf106/TatB line the channel wall, exposing their charged and hydrophilic face to create a hydrophilic channel wall [7]. This proposed mechanism has precedent in the amphipathic helix-containing antibacterial peptides, which open aqueous pores in membranes by inserting in pairs or higher oligomers [82,83]. Such surface seeking peptides possess a combination of average hydrophobicity (H) and hydrophobic moment (μ_H) that distinguish them from other helical peptides [83,84]. Analysis of the predicted amphipathic helices of Hcf106 and Tha4 by the method of Eisenberg et al. [84] shows that they cluster in the same region of an H vs. μ_H plot as surface seeking peptides (Cline, unpublished). However, if insertion of the amphipathic helices occurs, it must be induced by the presence of precursor and the pH gradient because in resting state thylakoids, the amphipathic helices of Hcf106 and Tha4 are accessible to externally added protease [52,61]. A ΔpH-induced change in Hcf106 and/or Tha4 conformation or aggregation state would most likely result from protonation/deprotonation of the conserved transmembrane glutamates in these proteins. Protonation-induced conformational changes in membrane proteins have previously been described. One example is the c subunit of the F_O portion of the ATP synthase, where protonation–deprotonation of a transmembrane aspartate coincides with conformational changes that drive rotation of the gamma/epsilon stalk of F_1 [85].

3. Perspective

Preliminary studies imply that the thylakoid Sec system operates similarly to the bacterial system.

Outstanding questions include whether there are other components involved in Sec transport, whether the cpSecYE translocon complex functions in the integration of thylakoid membrane proteins, many of which are plastid encoded but only recently have attracted much attention, and whether cpSecYE functions in concert with the proposed cotranslational cpSRP54 protein for insertion of plastid-encoded multispanning integral membrane proteins. On the other hand, much remains to be elucidated concerning the ΔpH-dependent/Tat protein transport systems. Although recent advances in describing the components, their organization in the membrane, and their involvement in precursor recognition are providing clues as to the operation of the system, much of proposed mechanism of translocation is pure speculation. Future endeavors will focus on the identity of the binding site for signal peptides in the cpTatC–Hcf106 complex, on whether Tha4 does in fact assemble with the cpTatC–Hcf106 complex to form an active translocon, on the structure of such a translocon, and on the mechanism by which a pH gradient can be transduced into mechanical energy for movement of the precursor protein. Another highly intriguing question is how the ΔpH-dependent machinery senses the size of precursor in order to adjust the diameter of the channel. It is possible that the above-mentioned 'proofreading' capability of these systems is part of such a size- and shape-sensing mechanism. The answers to these questions will assuredly result from the synergistic investigations of both the thylakoid system and the bacterial system.

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